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A MEMBRANE FILTER-FLUORESCENT ANTIBODY METHOD FOR DETECTION AND ENUMERATION OF BACTERIA IN WATER. RAPID IDENTIFICATION OF MICROBIAL POPULATIONS IN WATER

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A Membrane Filter-Fluorescent Antibody Method for Detection and Enumeration of Bacteria in Water

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A method is described for the combined use of membrane filter and fluorescent antibody techniques to detect and quantitate bacteria in water. The method has been tested as a means of detecting Escherichia coli presence in water supplies and has been compared with standard methods as to sensitivity and time required. Limitations have been discussed as well as the potential of the method to rapidly detect pollution of water supplies.

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The use of membrane filters with differential media preparations which allow direct specific determination of coliforms has been well established as a means to determine sanitary quality of water (9). In general, these methods require 18 to 24 hours for quantitative counts.

Fluorescent antibody techniques have been used for the identification of microorganisms from various sources. These techniques, however, require precise microscopic methods, particularly when dark-field is used. Danielsson (3) and Danielsson and Laurell (4) reported a membrane filter method for the demonstration of bacteria by fluorescent antibody techniques. That method employed filters mounted under the high power objectives of a microscope thus requiring manipulation of the membranes, and mathematical calculation for quantitation. Carter and Leise (2) had earlier reported a similar method for enumeration of anthrax colonies.

In water pollution, more rapid detection and enumeration of specific contaminating organisms may be of prime importance. For this reason, the following study was initiated to make use of the advantages of the membrane filter methods for concentration and growth of bacteria, and

fluorescent antibody techniques for early and rapid identification and enumeration of bacteria with the use of low power, large field microscopy.

METHODS AND MATERIALS

Production of Antiserum: Antiserum to eleven strains of Escherichia coli was produced in rabbits essentially by the methods described by Edwards and Ewing (5). Serum was used to identify specific E. coli strains or selectively pooled to give a polyvalent serum.

Conjugation Procedure: The sera were conjugated with fluorescein isothiocyanate by the methods described by Spendlove (8), and further purified after conjug· .ion by means of a DEAE cellulose column. The final conjugate was checked for purity and absence of albumin by cellulose acetate strip electrophoresis. Serum was stored at -15°C. in stocks of 10 ml/ml protein. Protein determinations were thawed and diluted prior to use to a protein concentration of 1 mg/ml.

Filter Equipment: Millipore black, gridded filters (HABG 047) in glass filter bases and funnels were used for this study. White filters and filters from other sources autofluoresce under the conditions of illumination and mask the

the specific fluorescence.

Fluorescence Microscopy: Darkfield preparations were observed with a Nikon SKE microscope fitted with an ultradark-field condenser (NA 1.50). Illumination was provided by a Bausch and Lomb Illuminator employing a 200 watt mercury arc lamp. Green, neutral density filters were used for illumination with visible light, and Corning 5-58 or 7-60 filters were used for ultraviolet illumination. Barrier filters were either the appropriate Y-8 or T-2, or the yellow filter supplied with the Nikon equipment. These filter combinations are equivalent to the UG-1 - GG9 or the BG-12 - PG1 systems currently used in many laboratories.

With the dissecting microscope, overhead illumination was accomplished by reflecting the light from a mirror supported above and to one side of the field. The total distance from the ar to the filter is 9 inches.

Fluorescent Antibody Slide Tests: In order to test each conjugated antiserum, the appropriate E. coli strain was fixed on a slide with acetone, followed by a wash with phosphate-buffered saline pH 7.5 (PBS) and overlayered with a drop or two of normal rabbit serum according to the suggestions of Bergman

et al. (1). The cells were then overlayered with the specific labelled antiserum or the pooled antisera and placed in a humidity chamber at room temperature for 20 minutes. The antiserum was washed gently by placing the slide in a coplin jar for three, 2-minute rinses in PBS, followed by a wash with carbonate-bicarbonate buffer, pH 9.0 and finally mounted under a cover slip with glycerol mounting fluid buffered to pH 9.0 with carbonate-bicarbonate buffer (9:0) as suggested by Pital et al. (7). Slides were observed for specific staining by using as controls. Only serum which gave 4+ staining was used in further tests.

Membrane Filter-Fluorescent Antibody Method (MFFA)

Water from a small pond was used to provide background organisms for controls and as a population from which to select specific E. coli strains which were added to samples of the water. The pond was monitored for three months using procedures outlined in Standard Methods (9), with no evidence of E. coli pollution. Predominant organisms were Bacillus species and Flavobacterium species.

Water was filtered through the membrane and the filter transferred to trypticase soy agar to allow growth of the

bacteria at 35°C. Colonies could be observed as early as five hours; however, for determination of this method, twelve hours was routinely used for incubation times.

At twelve hours, the filter was placed back on the filter funnel apparatus. The membrane was overlayed with 1 to 2 ml of pooled normal rabbit serum for 5 minutes at room temperature. This serum was removed through the membrane by applying negative pressure. Colonies were then overlayed with specific labelled antiserum and allowed to remain in contact with the serum for 15-20 minutes. Labelled serum was pulled through the filter by negative pressure and the colonies were first washed gently with 10 to 15 ml of PBS and finally with glycerol mounting fluid (pH 9.5). The membranes were observed immediately using a dissecting microscope (10X magnification) and total counts made under visible light, followed by a count of colonies fluorescing with a green color under UV illumination.

RESULTS

Using the techniques as described above, experiments were designed to test the efficiency of the membrane filter fluorescent antibody (MFFA) methods to accurately detect the

presence of varying numbers of fecal Escherichia coli in water samples. To 100 ml. of pond water, 10^3 E. coli ATCC 11303 cells were added. To a separate 100 ml. sample, the same number of non-fecal E. coli cells were added. Each preparation was then filtered through separate membranes in amounts of 1.0 ml., 0.5 ml., and 0.1 ml. The membranes were cultured on m-Endo and m-FC, and trypticase soy agar was used for fluorescent antibody staining. Results are shown in Table 1.

There appears to be little to choose between the methods in efficiency of detection of fecal Coliforms in a mixture of organisms in natural waters. The m-Endo and m-FC tests require 18-24 hours. These fluorescent antibody tests were done after 12 hours incubation.

To further test the MFFA method for detection and enumeration of coliforms in water, additional comparisons were made with the use of m-FC medium, and with the MPN determination for the water samples. Results are shown in Figure 1. The results with either method are seen to be well within the confidence limits of the calculated MPN. Again the major difference in the two methods has been shown to be the time required for the test.

Discussion

Repeat tests have indicated the reliability and specificity of the MFFA method for detection of fecal coliforms in water supplies. The advantage of the method is the short time required for completion. Although the work reported here was done at 12 hours for the purpose of standardization of reagents and techniques, additional tests have indicated that this time may be futher reduced. For the detection of coliforms, the time required is an extremely important factor which would be of benefit in the testing of water supplies for public health purposes.

In addition, the MFFA method has the advantage of being adaptable to the study of any bacterial species, and could therefore be used in a variety of pollution studies, and in the study of microbial populations of other interest in water sources.

The adaptation for the use of lower magnification, while requiring somewhat longer than the method of Daniellson (3, 4), has the advantage of allowing direct counting of the entire filter and eliminates the necessity for calculations. Critical factors in this method are: (1) filters must be washed during

staining by pulling solutions through the membrane keeping negative pressure on the filter at all times; (2) the glycerol mounting fluid must be used to fix the colonies, thus preventing dryness while enhancing fluorescence; and (3) once exposed to ultra violet light, the membranes must be rapidly counted before the intense illumination bleaches fluorescence from the stained colonies.

SUMMARY

A technique which employs use of non-fluorescing membrane filters and specific FITC-labelled antiserum has been successfully used in the identification and enumeration of known species of E. coli which have been added to natural populations of bacteria found in water. The quantitative results compared favorably with tests described in the standard methods. The use of a dissecting microscope with an external lighting arrangement provided a simple requirement for equipment. This method would appear useful in monitoring specific bacterial types from waters which were being monitored for specific pollution.

Acknowledgements

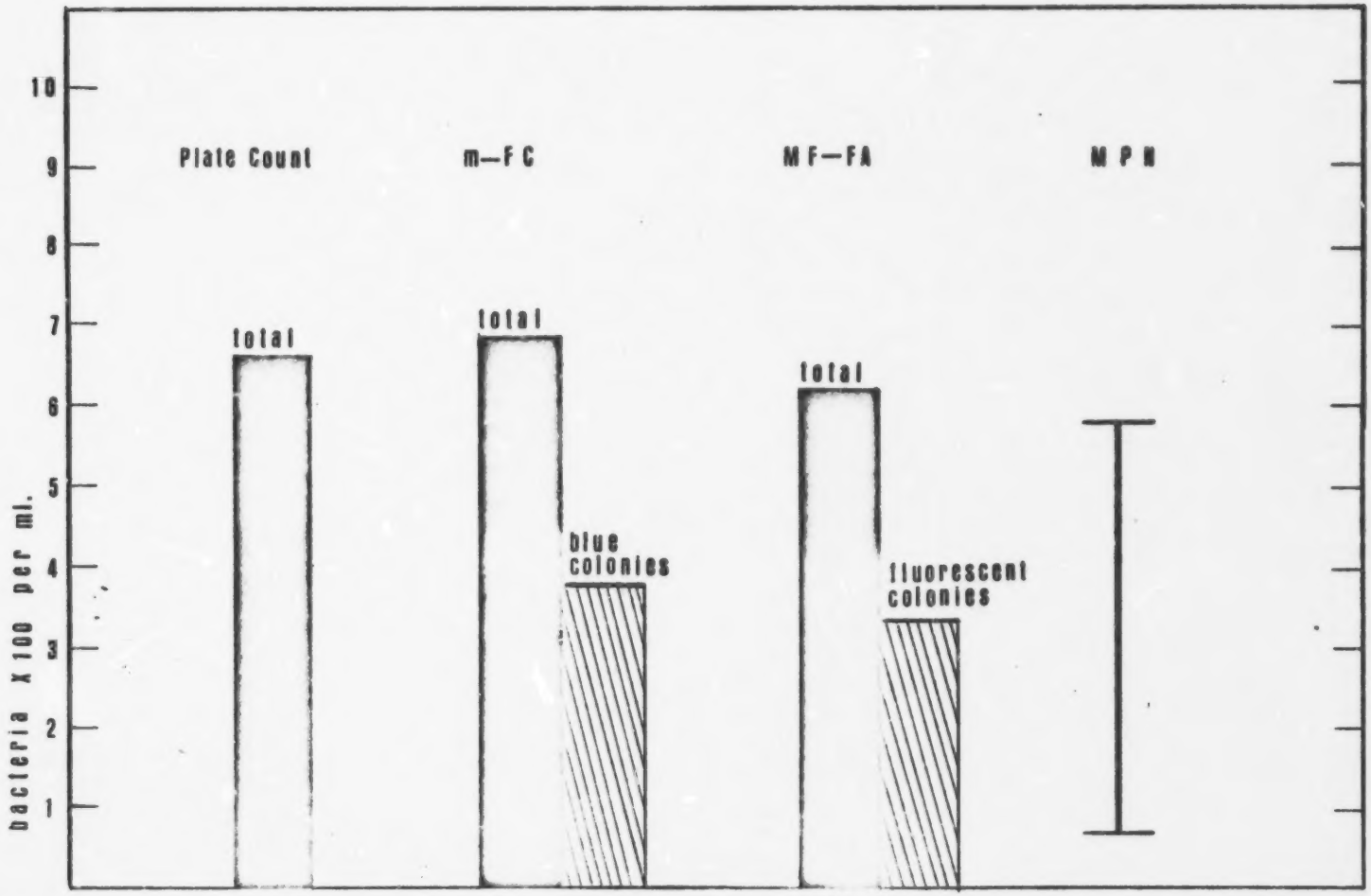
This work was supported by Grant WPD-146 from the Federal Water Pollution Control Administration, US Department of Interior.

Table 1. Comparison of fecal Escherichia coli Detection Efficiency of three methods

water sample tested	Amount, ml	Number of Colonies					
		m-Endo		m-FC		MFFA	
		green sheen	Total	Blue	Total	Fluorescent	Total
non-fecal	0.1	0	5	0	0	0	95
	0.5	0	7	0	0	0	500
	1.0	4	134	0	0	0	TNTC*
fecal	0.1	9	12	5	5	8	12
	0.5	17	21	9	10	11	16
	1.0	28	35	28	29	27	32

*Too numerous to count.

CORRELATION OF THREE TESTS FOR ESTIMATION OF COLIFORMS IN WATER



WPC 146-02

EFFECTS OF IMPOUNDMENTS ON WATER QUALITY IN THE SOUTHWEST

Discussion —

BACTERIAL CYCLES AND WATER QUALITY

by DR. RUFUS K. GUTHRIE*
Professor of Biology
North Texas State University
Denton, Texas

IN the determination of bacterial counts for the detection of cycles of presence in a large body of water, one must take into account several sources of error. One occasional happening which may produce major dislocations of bacterial presence is a period of heavy rainfall, producing extensive runoff into the water impoundment. Such drainage increases the total bacterial count for a period of one to two weeks and must be taken into account when looking for bacterial cycles which may occur in the water.

After studying bacterial presence in one water impoundment for a period of 1½ years, we have come to the conclusion that this influx of bacterial population does not alter the overall pattern of cycles which may be present. Total counts are increased by the runoff, and relative numbers of different groups are increased in approximately equal proportions. Following all counts drop off proportionately.

The occurrence of cycles of bacterial presence can be detected if one accounts for such periodic disturbances. In Figure 1 we show the results of consecutive periodic counts of bacteria over a period of approximately 15 months. In this figure we can see the higher counts occurring at times when

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*Read before Southwest Section, AWWA Convention held at Oklahoma City, Okla., Oct. 1967.

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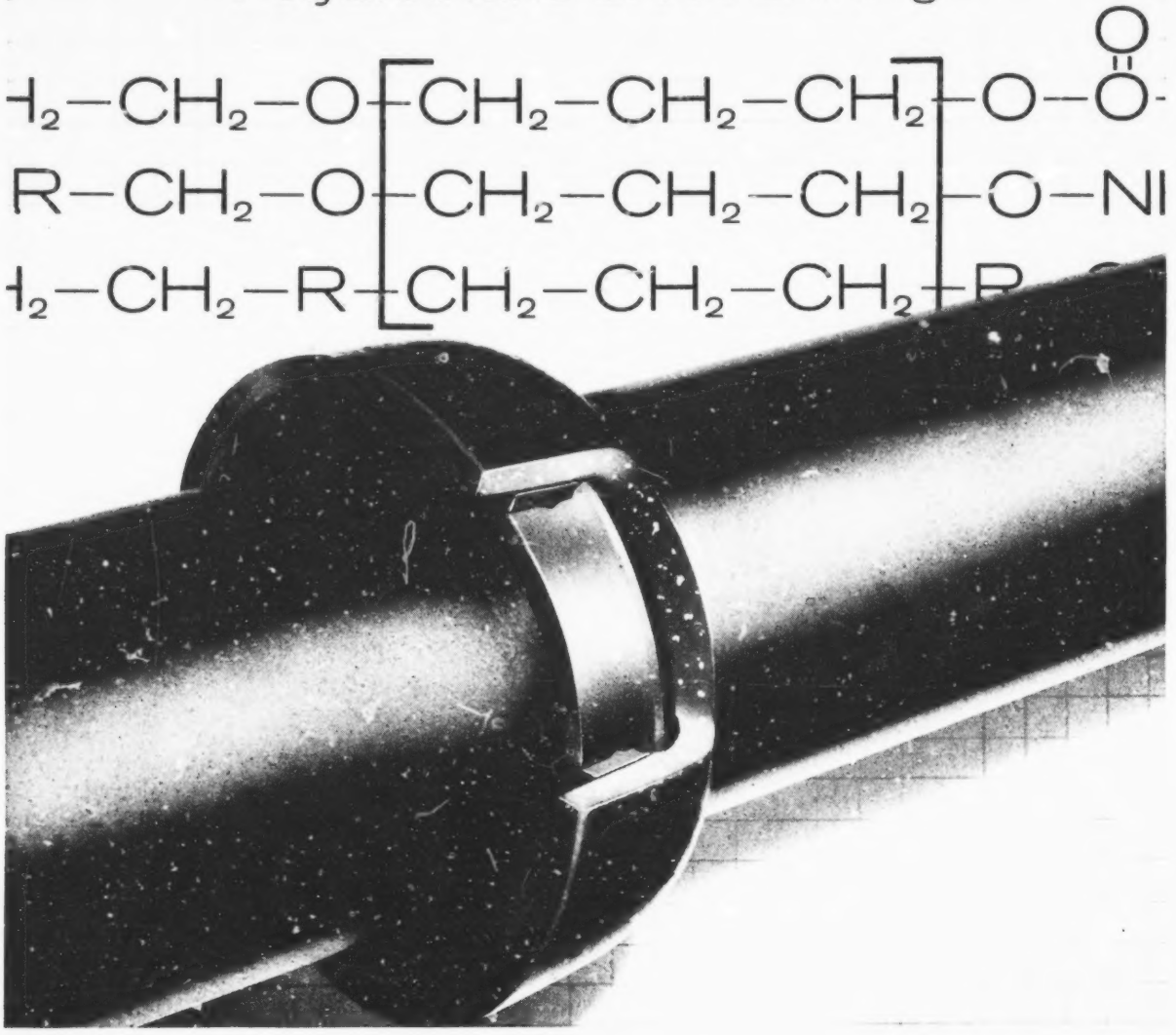

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tion of any other group. A cyclic balance is observed in this particular ecosystem.

In the water as pollutants, we may expect to find included the following assortment of organic chemicals: carbohydrates, hydrocarbons, and various assortments of proteins or protein source compounds. *Bacillus, Aerobacter, Micrococcus, Sarcina, Gaffkya,* and various strains of *Escherichia* are capable of fermentation of a wide variety of carbohydrates. This fermentation will be accompanied by the production of an assortment of chemical by-products including some of the essential growth factors, which will serve as nutrients for other microflora.

Hydrocarbons, not expected in large quantities here will be metabolized by a variety of bacteria, including *Pseudomonas* species and various soil forms. Products of this metabolism will in turn be used by other microorganisms.

Gram negative bacilli, *Brevibacterium* and others will metabolize proteinaceous materials and thus will assist in breakdown of cell bodies. Many soil forms also assist in this process, sometimes with the production of ammonia, hydrogen sulfide, and putrefactive odors. However, with a balanced micropopulation, the metabolism here should be relatively complete and these obnoxious products will thus be avoided.

With this assortment of metabolic activities and the maintenance of cyclic bacterial population presence, the water quality is maintained at a level which makes it desirable for human uses. This quality involves both chemical and bacterial factors. As long as the quality remains good, we can expect the maintenance of bacteriological cycles, which maintain the chemical quality. If we overload the chemical quality in any direction we will permit the predominance of certain bacterial groups to the exclusion of others, and eventually in this manner, will further increase the imbalance and produce water unfit for human uses.

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Weight of Bondstrand/du Verre pipe is approximately 1/5 to 1/8 of equivalent steel sizes, depending on design requirements and pipe wall thickness. To provide complete design flexibility in large diameter systems, Amercoat makes available virtually all standard ASA-type fittings. Joints are made and fittings connected, using simple adhesive-welding techniques.

The new large diameter FRP pipe is manufactured by filament-winding, but incorporates a 110 mil SPI-type liner. Filament winding provides the strength needed in large diameters without the prohibitive wall thickness and weight normally incurred using hand lay-up techniques. Result is design hoop tensile strength of 70,000 psi, design axial tensile strength of 70,000 psi and design axial flexural strength of 40,000 psi. The SPI-type inner wall provides resistance to more than 300 major industrial chemicals.

we expect heavier rainfall—June '66, December '66, February '67, June '67. To detect cyclic presence then we must consider relative proportions in group populations, shown in Table 1.

In consideration of these data we have arbitrarily designated any presence above 5% as being a major segment of the total population. If this is done and we look for seasonal patterns, we can designate the cool months as a time of high presence of gram negative organisms. (These include the *coliaerogenes* groups, *Alcaligenes*, colon-like organisms, *Achromobacterium*, and soil forms such as *Azotobacter* and *Rhizobium*.) The warmer months show a predominance of gram positive organisms—(*Bacillus, Brevibacterium, micrococci, Sarcina, Gaffkya*, and *Corynebacterium*).

Groups which show no pattern of presence are gram negatives—*Flavobacterium* and *Pseudomonas*, and gram positives—*Brevibacterium*. The fungal presence is one which must be considered with some skepticism, because of the nature of the organisms, and particularly because of the ease of spread and contamination with fungal spores. Apparently, the fungal presence parallels closely the presence of bacilli. Whether this is meaningful or not, we cannot say at this time.

In these results we cannot assign great significance to the continued high presence of *Bacillus* species, since these organisms were added to the lake in the warm months. We can say that these bacteria were able to maintain a high presence in association with the other micro populations of the water; however, a permanent predominance was not established. One feature of these data is noteworthy, in that an extremely high presence of *Bacillus* is in most cases accompanied by a high presence of other gram positives, and a drop in gram negatives of all types. The reverse is true of a high presence of gram negatives of any variety. A high presence of *Flavobacterium* is always accompanied by a high presence of other gram negatives with these periods then being followed by shifts to high presence of bacilli and other gram positives.

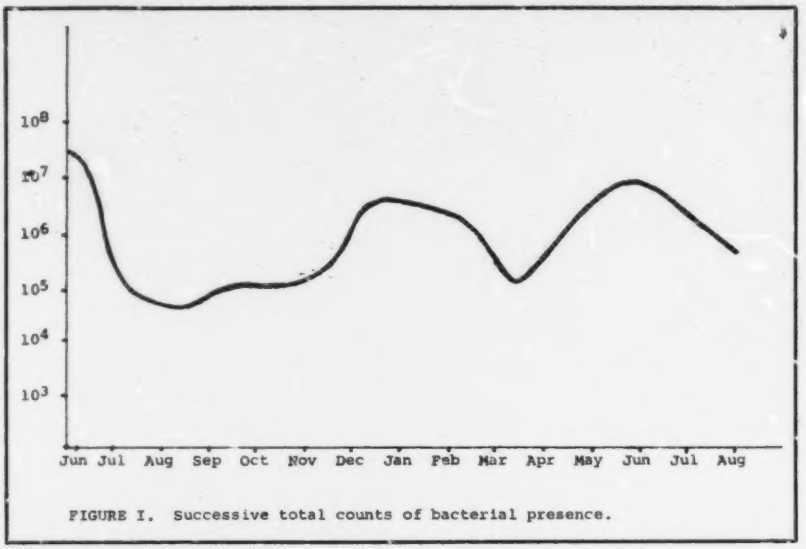

FIGURE I. Successive total counts of bacterial presence.

These cylic shifts do not appear, nor indeed to the relative proportions of bacterial presence appear to be greatly affected by the influx of water in high runoff periods which can be detected by the highest total counts. We do detect an increase in the presence of fungi following an influx of runoff.

We have not yet mentioned the Actinomycetes. These organisms show no particular pattern of presence. We do note that the presence was higher in July and August 1966 than at any other date, and the summer of 1967 did not show a repeat of this increased incidence in that the presence of Actinomycetes never reached a 5% level after February 1967. Yet relative presence figures of other bacteria of summer 1967 again approximated the figures of 1966.

With these data we may speculate on the chemical events which affect water quality. I think we must begin with the prediction that this water is of consistently good bacteriological quality. This conclusion is not based on the total absence of any proven pathogens or the absence of *E. coli*. Rather we would base this statement on the fact that we do have at all times a good mixture of bacterial populations, and that all members of this mixture are able to maintain continual presence. We have observed no loss of population segments for appreciable lengths of time, and we have observed no predominance of any population segment. The addition of one segment (*Baccilus*) did not allow the elimina-

"You'll catch on, but I think it would be safer if you'd forget about all you've learned about riding a motorcycle!"

Table 1. Percentage of bacterial groups in successive samples.

	1966								1967					
Month #	†6	7	8	9	10	11	†12	1	†2	3	4	†6	7	8
Bacillus	22	14	20					8			7	11	40	10
Fungi	34	10	40					8		7		6	20	10
Actinomyces		5	9	10					5					
Pseudomonas						11					5	10		
Flavobacterium	5	20	6		6		26			27	19	19		5
Brevibacterium	5	14				30		16				6		10
Gram - Rods	15	25	16	76	46	37	65	51		51	42	34		41
Micrococci	5	5	6	5	41		27						15	10
Other Gram +	5	6	5	8			5			5	22	12	20	10

†Periods of highest total bacterial counts.

WPD - 146 - 02 - 68

Rapid Identification of Microbial Populations in Water

North Texas State University
Rufus K. Guthrie, Ph.D.
Professor of Biology

November 1, 1967 - November 1, 1968

Prepared November 1, 1968

Summary Statement

The second year of this study has been used to: (1) Complete the collection of data to determine the relative presence of microbial populations in a fresh water impoundment as a continuous study for two years; (2) Complete the development of the fluorescent antibody-membrane filter methods for identification and enumeration of microbial populations in water; (3) to produce antisera specific for Escherichia coli strains and to conjugate these antibodies with fluorescent dyes; (4) to produce and conjugate antisera specific for Streptococcus fecalis strains; (5) to produce and conjugate antisera for Bacillus cereus strains; (6) to run tests with these antisera for the determination of (a) the sensitivity of this method in detection of small numbers of specific bacteria in water samples and (b) to reduce the required time of incubation of test membranes in order to make these methods more practical.

Statement of Progress

The two year study for determination of relative presence of microbial populations in Lake Hefner, Oklahoma City, was completed in June, 1968. Copies of a manuscript including this work are included. This manuscript has been submitted for

publication in Environmental Science and Technology and is currently being reviewed.

The development and refinement of the fluorescent antibody-membrane filter methods has been completed in that our methods have provided accurate and repeatable results in test studies when compared to other standard methods requiring longer times. Our methods have just been written in manuscript form, copies of which are included, and will be submitted to Applied Microbiology this month.

Considerable stocks of antiserum have now been produced and conjugated for 12 strains of E. coli, 4 strains of S. fecalis, and are in production for 4 strains of Bacillus cereus. While some amounts of antisera have been produced for other bacteria, work has been concentrated on the three species above because the investigators feel that these species offer the best potential for useful studies of pollution problems.

In work with E. coli using this technique the problems of strain specificity and cross reactions reported in the 1967 report, have been largely overcome, and as is reported in the enclosed method manuscript, our reagents can be used to differentiate between fecal and non-fecal strains of E. coli. This specificity

in these methods reaches the quality desired for this work.

Currently in process, are repeated tests to establish the lower limits of bacterial detection using either E. coli or S. fecalis as test organisms, and to establish the minimum incubation time for filters to obtain colonies detectible by this method. With either organism, repeated tests have detected single colonies in mixtures of other bacteria on filters. This detection indicates the specificity of the antiserum and the lack of cross reactivity of the labeled antibody with those bacteria. In our tests we have used 100 ml water samples for filtration and the method can therefore detect as few as one viable bacterium/100 ml undiluted sample.

In time studies, E. coli colonies become visible at approximately 5 hours when filters have been incubated on Trypticase Soy Agar plates. At this time, the colonies are somewhat more easily washed off in the staining processes, however, with careful attention to techniques, the colonies can be specifically stained at this age. Repeat tests are being performed currently to determine the reliability of staining, detection and enumeration at this incubation time.

S. fecalis colonies on filters incubated on Trypticase Soy

Agar plates become visible at 6 hours incubation. The reliability of the method at this incubation time is being determined.

With the completion of the above determinations, these methods will be used to evaluate the efficiency of sewage treatment as measured by the presence of fecal E. coli in raw sewage and in effluent at various distances from the intake of effluent in a large water impoundment. Experimental designs and preliminary testing for determination of dilutions required are currently underway.

The project director, Rufus K. Guthrie, Ph.D., spent full time on the project for six weeks during the summer and has spent approximately 15% of the remainder of his time on this project. Dr. J. K. G. Silvey has not been paid by the project, however, he has devoted considerable time in consultation and supervision of specimen collection for this work. Other people paid by this grant have been graduate students on a part-time basis.

Reports and Technical Articles

1. "Bacterial Cycles and Water Quality." Rufus K. Guthrie. Southwest Water Works Journal. 50:54-56. 1968

2. "Fluorescent Antibody-membrane filter method for specific enumeration of bacteria in water." D. J. Reeder, and Rufus K. Guthrie. Reported to North Texas Biological Society, Fort Worth, (4-27-68) and to American Society for Microbiology, Detroit, Michigan, May 5-10, 1968. Abstracted in Bacteriological Proceedings, 68: 34. 1968.

3. "Seasonal distribution of bacteria in a fresh water impoundment." R. K. Guthrie, J. C. Stiles, J. K. G. Silvey and K. Dickson. Submitted to Environmental Science and Technology. August, 1968.

4. "A membrane filter-fluorescent antibody method for detection and enumeration of bacteria in water." R. K. Guthrie and D. J. Reeder. To be submitted to Applied Microbiology. November, 1968.